AWARD NUMBER: W81XWH-13-1-0427

TITLE: Organtropic Metastatic Secretomes and Exosomes in Breast Cancer

PRINCIPAL INVESTIGATOR: Dr. David Lyden, MD, PhD

CONTRACTING ORGANIZATION:
Weill Medical College of Cornell University, New York, NY 10065

REPORT DATE: October 2014

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

1. REPORT DATE	2. REPORT TYPE	3. DATES COVERED
October 2014	Annual, year 1	27 Sep 2013 - 26 Sep 2014

4. TITLE AND SUBTITLE

Organtropic Metastatic Secretomes and Exosomes in Breast Cancer

5a. CONTRACT NUMBER

5b. GRANT NUMBER
W81XWH-13-1-0427

5c. PROGRAM ELEMENT NUMBER

6. AUTHOR(S)

David Lyden, MD, PhD, Hector Peinado Selgas, PhD, Ayuko Hoshino, PhD, Irina Matei, PhD
Email: dcl2001@med.cornell.edu

5d. PROJECT NUMBER

5e. TASK NUMBER

5f. WORK UNIT NUMBER

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Weill Medical College of Cornell University
407 East 61st S Street Rm 106 1st Floor
New York NY 10065-4805

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

11. SPONSOR/MONITOR'S REPORT NUMBER(S)

12. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

13. SUPPLEMENTARY NOTES
N/A

14. ABSTRACT Metastasis to distant vital organs (bone, lung, brain) is the most devastating feature of breast cancer. We proposed to extend our current integrative genomic, proteomic and transcriptomic analysis on the crosstalk between breast cancer cells and bone and lung microenvironments during organ-tropic metastasis. An understanding of secreted metastasis regulators (extracellular proteins, cell-free nucleic acids and small vesicles –exosomes-) has tremendous potential to improve the diagnosis, prognosis and treatment of breast cancer. We hypothesized that tumor and stromal cells communicate via secreted and exosomal proteins and miRNAs to promote organotropic metastasis. Therapeutic disruptions of these communication pathways may significantly increase diagnostic options, improve treatment efficacy and survival of breast cancer patients. The **objectives** of our proposal are to comprehensively analyze secreted and exosomal proteins and miRNAs that are regulators of bone and lung metastasis, to characterize their function in mediating tumor-stroma interactions, and to determine the potential of utilizing such circulating factors as biomarkers and therapeutic targets. Our specific aims are: 1) Identification and functional characterization of secreted factors promoting bone and lung metastasis; 2) Determination of the role of exosomes in metastatic progression and niche formation; 3) Clinical analysis of metastatic secretome and exosomes.

15. SUBJECT TERMS
breast cancer, exosomes, organ-specific metastasis

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF PAGES	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			USAMRMC
Unclassified	Unclassified	Unclassified	Unclassified	11	19b. TELEPHONE NUMBER *(include area code)*

Table of Contents

DOD Grant/Contract (Award Number W81XWH-13-1-0427)

YEAR 1 RESEARCH REPORT

Grant Title: Organ-tropic metastatic secretomes and exosomes in breast cancer

INTRODUCTION:

Background: Over 90% of breast cancer deaths are caused by the metastatic spread of tumors to vital secondary organs, including bone and lung. Pathogenesis of metastasis is likely mediated by intercellular communication between tumor cells and the stromal microenvironment. In addition to direct cell-cell contact, many of such tumor-stromal interactions occur via secreted factors, such as growth factors, cytokines, cell-free nucleic acids and small vesicles called exosomes. A comprehensive understanding of secreted molecular mediators of tumor-stroma interactions in organ-tropic metastasis of breast cancer to bone and lung has tremendous potential impact on improving the diagnosis, prognosis and treatment of breast cancer. We postulated that tumor and stromal cells communicate via secreted and exosomal proteins and miRNAs to promote organotropic metastasis. Therapeutic disruptions of these pathways may significantly improve disease diagnosis and prognosis, as well as reducing the morbidity and mortality associated with metastasis. The **objectives** of our proposal are to comprehensively identify secreted and exosomal proteins and miRNAs that are functional mediators of bone and lung metastasis, to characterize their functional mechanisms in mediating tumor-stromal interactions, and to determine the potential of utilizing such circulating factors as biomarkers and therapeutic targets.

Summary of the tasks/aims proposed and achievements:

Task 1: Identification and functional characterization of secreted factors promoting bone and lung metastasis (Months 1-36).
Given the paucity of studies on secreted proteins and miRNAs with functional relevance in metastatic organ-tropism we are currently analyzing secretomes and extracellular miRNAs from lung (**Lyden laboratory**) and bone metastatic breast cancer cells (**Kang Laboratory)**.

Task 1a: Identify differentially secreted miRNAs associated with bone-tropism of breast cancer cells **(Months 1-36)**. **Dr. Kang's group is responsible for this task.**

Task 1b: Identify differentially secreted proteins and miRNAs associated with lung-tropism of breast cancer cells **(Months 1-36)**.

- We are currently optimizing RNA isolation from lung metastatic cancer cells, performing RNA-Seq at the WCMC Genomics Facility. The data will be deconvoluted and will undergo bioinformatic analysis at the WCMC Institute for Computational Biology, under the supervision of Dr. Olivier Elemento. We used

the parental MDA-MB-231 as well as sublines with high and low lung metastatic tropism and the MCFCA1h (poorly metastatic) and MCFCA1a (highly metastatic) pair of human breast cancer cell lines. We also plan to isolate miRNAs from the 4T1 series of mouse mammary tumor cell lines with progressively higher lung metastatic abilities and are planning on sequencing these **(Months 1-6 of year 2)**.

• Once top lung tropic secretome miRNA and proteins are identified, we plan to test the detection of secretome candidate proteins/miRNAs in animal models: healthy, primary tumor bearing, spontaneous and experimental lung metastasis. 40 nude and Balb/c mice will be used **(Months 6-12 of year 2, as soon as ACURO approval is obtained)**.

• We are currently working on methods to optimize the comparison between secreted and exosomal miRNA/proteins isolated from lung-tropic and parental control cell lines.

Outcome and Milestones: We expect to identify distinct secretome miRNA/protein profiles (Lyden) of breast cancer cell lines with differential lung metastatic capabilities whose pathological relevance can be validated in animal models of lung metastasis. We plan to publish the results based on the lung-tropic secretome proteomic analyses at the end of the third year of funding.

Task 1c: Stroma-derived miRNAs as biomarkers and potential therapeutic targets **(Months 1-24). Dr. Kang's group is responsible for this task.**

Task 2: Determination of the role of exosomes in metastatic progression and niche formation (Months 1-48). ***Task will be performed by Dr. Kang (bone metastatic exosomes & functional analysis), Dr. Lyden (lung metastatic exosomes & functional analysis), Dr. Garcia (proteomics), Sequencing Core Facilities (RNA-Seq).***

Based on our previous studies, tumor-derived exosomes can promote metastasis by transfer of functional factors. This aim will analyze exosomal proteins and miRNAs released from stromal and bone and lung metastatic breast cancer cells to identify metastasis regulators.

Task 2a: Identify differences in exosomal protein/miRNA composition between highly and poorly bone metastatic breast cancer cells and determine the function of the candidate exosomal bone metastasis regulators **(Months 1-48)**. **Dr. Kang's group is responsible for this task.**

Task 2b: Identify differences in exosomal protein/miRNA composition between highly and poorly lung metastatic breast cancer cells and determine the function of the candidate exosomal lung metastasis regulators **(Months 1-48)**.

• We isolated and characterize exosomes from cancer cells with different lung metastatic capabilities, and analyzed protein and miRNA contents by mass-spectrometry and Ref-Seq, respectively. RNA-Seq of exosomal miRNAs was performed at the WCMC Genomics Facility. The data is currently being deconvoluted and undergoing bioinformatic analysis at the WCMC Institute for Computational Biology, under the supervision of Dr. Olivier Elemento. We used

the parental MDA-MB-231 as well as sublines with high and low lung metastatic tropism and the MCFCA1h (poorly metastatic) and MCFCA1a (highly metastatic) pair of human breast cancer cell lines. Preliminary Ingenuity pathway analysis revealed that the top pathways represented by the proteins packaged in lung tropic exosomes compared to the parental line were: glycolysis and remodeling of epithelial adherens junctions. We also plan to identify exosomal miRNAs and proteins from the 4T1 series of mouse mammary tumor cell lines with progressively higher lung metastatic abilities. The exosomal miRNA sequencing and proteomics data will be compared and top candidates present in both datasets will be selected for further functional analysis **(Months 1-6 of year 2)**.

FUTURE DIRECTIONS FOR YEAR 2:

• We plan to test the detection of exosomal proteins/miRNAs in plasma of animal models: healthy, primary tumor bearing, and bone metastases bearing mice **(Months 12-24, as soon as ACURO approval is obtained))**.

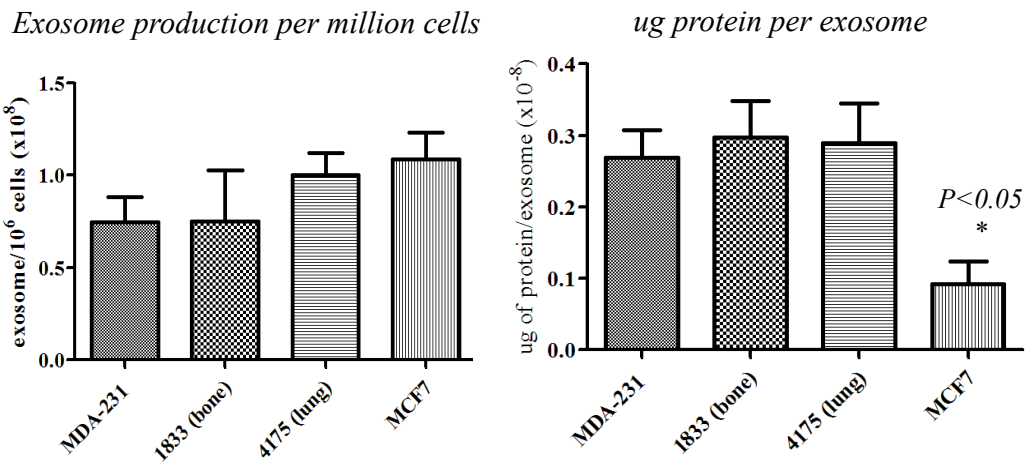
• We will create KD/OE lines of candidates and evaluate their metastatic capabilities. We will use exosomes from generated cell lines to treat mice bearing primary tumors and bone metastases, and evaluate their effects **(Months 12-36)**.

• We will label exosomes with dyes or epitope tags and investigate the cell identity of target cells. We will then characterize the downstream effects induced *in vivo* in target cells following exosome uptake **(Months 12-36)**.

Task 2c: Investigate the functional role of stroma-derived exosomes in metastasis **(Months 12-24)**. **Dr. Kang's group is responsible for this task.**

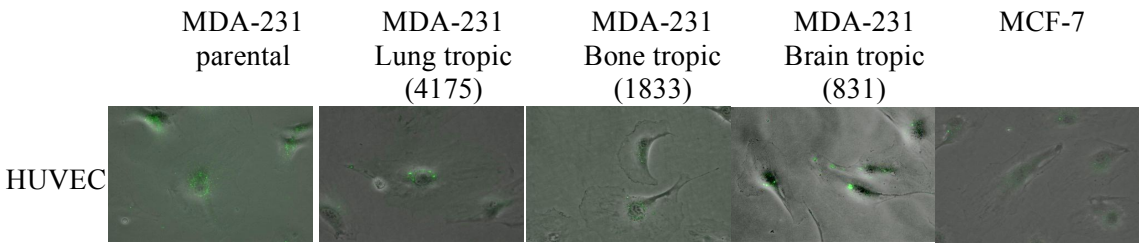
Outcome and Milestones: We have identified several candidate exosomal proteins and miRNAs from tumor and associated stromal cells whose manipulation changes the metastatic abilities of breast cancer cells. We expect to identify target cells and downstream effects of exosome uptake. Multiple papers reporting the role of exosomes in bone and lung metastasis will be published in years 2, 3 and 4.

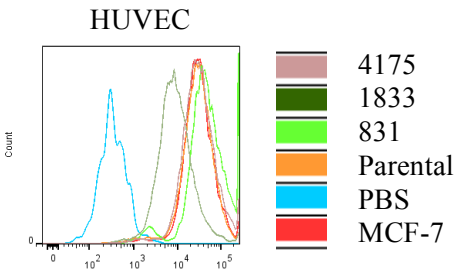
Figure 1. Analysis of exosome secretion in organ-tropic breast cancer models. The graph on the left represents exosome production per million BC cells. Cells were cultured for four days with exosome-free media followed by exosome isolation. Exosome numbers were quantified using Nanosight analysis. The graph on the right represents the amount of exosome protein, measured in micrograms by BCA assay per exosome. Exosome numbers were quantified using Nanosight analysis.



We have successfully isolated exosomes from both models (MCF-7 and MDA-231, as well as organ-tropic MDA-231 variants) and characterized their properties by Nanosight and mass spectrometry. Our data demonstrates that highly malignant models (MDA231 and the BC cell line variants with tropism to the bone (1833) and to the lung (4175) secrete similar amounts of exosomes compared to the non-metastatic cell line MCF7. Importantly, we have observed that the amount of protein per exosome in highly malignant MDA-MB-231 models is significantly increased suggesting that exosome cargo could be involved in BC cell malignancy (Fig. 1). We have used 3 different models tropic to the lung, bone or brain. Analysis of BC exosome cargo by mass spectrometry demonstrated that there is a proteomic signature associated with the metastatic organotropism of these cell lines (data not shown, data is in the bioinformatics pipeline, being analyzed). We have developed and improved the protocol for labeling of exosomes with fluorescent dyes. Our data show that exosomes can be fluorescently labeled using red and green PKH membranes dyes as well as using near-infrared fluorescent dyes (i.e. CellVue).

Figure 2. Breast cancer exosomes are efficiently uptaken by stromal cells.





We have initiated the analysis of tumor exosome uptake *in vitro*. For this purpose exosomes were first labeled fluorescently and incubated with fibroblast or endothelial cells (Fig. 2). We have used different models of stromal cells to perform these assays and incubated these cells with exosomes derived from parental MDA-MB-231 BC. Our data demonstrate the ability of these cell types to uptake BC exosomes. Specifically, we have analyzed the ability of NIH3T3 fibroblast to uptake exosomes (data not shown) and we observed that over 90% of cells are able to uptake exosomes over 24 hours of incubation with 10ug/ml BC exosomes. Similarly, our analysis in HUVEC endothelial cells demonstrate that over 98% of cells uptake exosomes efficiently (Fig. 2). These data support the idea that BC exosomes can be transferred to stromal cells and tracked *in vitro*.

To identify downstream effects of exosomal interaction with target cells, we treated WI-38 lung fibroblasts and HBEpC lung epithelial cells with lung-tropic MDA-MB-231-derived exosomes. Since upregulation of S100 genes in stromal tumor cells correlates with metastasis, we analyzed the expression of a panel of S100 genes in tumor exosome-educated WI-38 lung fibroblasts and HBEpC lung epithelial cells. Multiple S100 genes (S100A4, -A6, -A10, -A11, -A13, and -A16) were upregulated more than five-fold upon WI-38 lung fibroblast treatment with lung-tropic MDA-MB-231-derived exosomes. Interestingly, expression of the S100 genes tested remained unchanged in HBEpC cells treated with lung-tropic MDA-MB-231-derived exosomes (data not shown), recapitulating the resident cell type-specific effects observed previously. In addition, we found that exosome-treated fibroblasts showed higher proliferation and migration rate compared to PBS-treated controls (data not shown). Collectively, these data indicate that exosomal integrins can regulate the pro-inflammatory effects of

certain S100 molecules in target cells, influencing the expression of genes implicated in tumor metastasis.

Upon receipt of ACURO approval for animal studies, we will analyze the *in vivo* biodistribution of exosomes isolated from organ-tropic cell lines. Moreover, we will combine exosome labeling with immunofluorescence studies using cell-type specific markers to identify the specific cells uptaking tumor exosomes in each destination organ. We will perform these approaches *in vivo* during the second year of funding.

Task 3: Clinical analysis of metastatic secretome and exosomes (Months 24-60). In collaboration with Dr. Bromberg (clinical sample collection and analyses) we have been accruing and isolating exosomes from plasma samples from breast cancer patients with metastasis to various sites (lung, brain, bone). Up to 7/27/2014, we had accrued 10 breast cancer samples with metastatic dissemination to specific sites. Once functionally relevant exosomal protein and miRNA candidates are identified, their levels of expression will be tested in these samples by RT-PCR (RNA) or ELISA (protein). We predict that due to their extracellular localization, secreted factors represent superior biomarkers and therapeutic targets.

Outcome and Milestones: We expect to validate candidates identified in Tasks 1 and 2 in patient samples. We expect that some factors will have diagnostic and/or predictive value. Those with function in metastasis may become potential therapeutic targets. These results will be published in several papers in year 5.

KEY RESEARCH ACCOMPLISHMENTS:

- We determined for the first time that the amount of protein per exosomes is increased in exosomes derived from parental MDA-MB-231 and organ-tropic variants, and that they can be efficiently labeled with fluorescent and near-infrared dyes.
- We have demonstrated that tumor-secreted exosomes can be efficiently uptaken by fibroblast, endothelial and bone marrow-derived cells, depending on their tropism.
- Importantly, we have analyzed the proteomic and miRNA content of exosomes isolated from organ-tropic BC models and developed a database of protein secreted in exosomes secreted by organ tropic breast cancer cells.

REPORTABLE OUTCOMES: Provide a list of reportable outcomes that have resulted from this research to include:

• Dr. Lyden has presented preliminary data from some of these studies in 10 national and international meetings this past year. The top five meetings were:

- San Antonio Breast Cancer Conference, San Antonio, Texas, USA, Dear December 10-13, 2013.
- AACR sponsored meeting on the tumor microenvironment in San Diego, California, USA, Feb 26 -March 1, 2014
- International Society of Extracellular Vesicles, Rotterdam, The Netherlands, April 30-May 3, 2014
- The TMEN NCI Meeting, June 10-12, 2014, Weill Cornell Medical College, New York, NY, USA (Dr. Lyden organized and hosted this meeting)
- International Metastasis Meeting in Heidelberg, Germany, June 27 - July 2, 2014

• Dr. Peinado has presented as invited Speaker at the following meetings:

- V European Melanoma Workshop, Marseille, France, July 2013.
- Symposium on exosomes and microvesicles, UIMP, Spain, September 2013.
- University of Vermont, USA, October 2013.
- Extracellular Vesicles as Therapeutics. Rhode Island, USA, June 2014.
- V GEM and 6th European Melanoma Workshop. Canary Island, Spain, July 2014.

CONCLUSION:

We have confirmed that BC exosomes can be efficiently labeled fluorescently and tracked *in vitro*. The biological data confirm that highly malignant, organ-tropic breast cancer cell lines secrete exosomes with increased amount of protein and can be transferred to specific types of stromal cells, depending on the tropism of the cancer cell line the exosomes were derived from.

The identification of molecules expressed on exosomes that could “address” exosomes to specific metastatic sites could predict metastatic niches and allow foreseeing metastatic spread of tumors and metastatic organ.

We propose to test this hypothesis during next year in animal models of organ-tropic breast cancer metastasis.

Impact: Our research will unveil novel secreted and exosomal proteins and miRNAs as functional regulators of long-range communications between metastatic tumor and stromal microenvironment. Moreover, due to their

extracellular localization, secreted factors represent superior biomarkers and therapeutic targets as they can readily enter body fluids where they can be non-invasively detected, targeted or restored. Thus, we expect this research to open up exciting novel avenues of clinical translation in early breast cancer and metastasis detection, prognosis and therapy. Therapeutic strategies may include, but not be limited to, monoclonal antibody for protein targeting, restoration of metastasis-inhibiting miRNAs and proteins, and modulation of signaling pathways activated by secreted proteins or targeted by miRNAs.

REFERENCES: Not applicable. No references are associated with this report.

APPENDICES: Not applicable. No appendices are attached to this report.

SUPPORTING DATA: Figures 1-2.